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CONTENTS

SUMMARY

We generated a novel SHIV (termed SHIV-pr) that possesses the HIV-1-derived protease (PR) gene in the corresponding position in the SIVmac genome. SHIV-pr is replication-competent in human and monkey $CD4^+$ lymphoid cell lines as well as rhesus macaque PBMCs. The viral growth of SHIV-pr was completely blocked in the presence of a peptide-analog PR inhibitor at the tissue culture level. When SHIV-pr was intravenously inoculated into two rhesus macaques, one monkey resulted in a weak but long-lasting persistent infection whereas the infection of another was only temporary. To enhance the viral growth competence by adaptation, we then passaged the virus *in vivo* from a monkey up to the fourth generation. The initial peak values of plasma viral loads as well as the set-point values increased generation by generation and reached those of a parental virus SIVmac. During the course of in vitro passages we detected two amino acid mutations A71V and V77I in the PR domain both of which were commonly found in the monkey plasma samples of the 2nd through 4th generations. When a medication using the contents of a Kaletra capsule (a mixture of two PR

inhibitors, lopinavir and ritonavir) was orally given to three SHIV-pr-infected monkeys for 4 weeks, plasma viral loads dropped to near or below the detection limit and quickly rebounded after the cessation of medication. No significant mutational changes were observed samples in the plasma from 2 monkeys except one mutation (L33V in the PR region) in a monkey before and after the medication. The results suggest that SHIV-pr can be used to evaluate PR inhibitors using monkeys.

INTRODUCTION

Acquired immunodeficiency syndrome (AIDS) is the disease showing various immunological disorders among which the destruction of immune system is a typical symptom in the patients. Since the first case report of the disease was documented by Gottlib et al. (1981), researchers such as virologists, immunologists as well as medical doctors immediately paid their great interest to this malady. Before long the disease was reported in 1983 to be caused by human immunodeficiency virus type 1 (HIV-1) as the etiological agent by Barre-Sinoussi et al..

In 1980s, AIDS was one of the most fearful diseases to the mankind mainly because of its high fatality and its tremendously speedy increase of the number of patients not only on the nation-wide but worldwide scales. Nonetheless, this disease has become a controllable sickness thanks to the development of various anti-HIV drugs. In early 1990s, there was a big step to fight against AIDS in which some nucleotide-analog compounds were found to specifically bind to the HIV-1 viral proteins (reverse transcriptase) and inhibit the viral proliferation. Administration of such compounds to

the patients as a therapy made it possible to suppress the disease progression in them. However, the use of a single drug alone easily allowed a quick appearance of a tolerant virus due to a highly easiness of the mutation of the viral genome, and thus the therapy with a single drug ended up as a failure. In the latter half of 1990s, a new therapy called highly active anti-retroviral therapy (HAART) was developed and introduced to the clinical field. Briefly speaking, HAART is the combination therapy using multiple anti-HIV drugs at the same time. The effect was dramatic and made it possible to suppress emergence of the drug-resistant virus over a long-term period. Therefore, a more or less optimistic perspective that HIV is no more a threat to the mankind has started to circulate among general populations, which is regretfully not true.

Although the effect of HAART is doubtlessly remarkable, the physical and monetary burdens to the patients who undergo this therapy are not easy at all. First, the patients need to take a great amount of drugs every day and cannot evade from the suffering of various side effects especially when they undergo long-term treatment. Second, the hospitalization is still costly for the patients of ordinary incomes. In addition, they are always facing a fear that emergence of a drug resistant virus may

occur any time and there is no effective way to stop the disease progression once it happens. The AIDS dementia is one of the symptoms of the disease. In case of this onset, the family members and the health care people supporting the patient are most likely to be enforced to fairly exhaustive degrees. Apart from the above-mentioned issues, establishment of the society that will be ready to fully support the patients still remain untouched. Receiving hospitalization denial and discrimination on nursing may not be a past story even at major hospitals.

Regarding to prevention of HIV infection, vaccine research and development have been conducted actively, but the result so far is not yet successful. Even if a vaccine which can succeed in prevention of new infections is in our hand, the people who already got infected with the virus cannot be cured. To fundamentally solve the problem of AIDS, it is necessary to combine a means of controlling the HIV infection and that of curing the disease. To do so, it is essential to evaluate more potentially efficatious drugs *in vivo* with the aid of animal model for AIDS. Because the results obtained at the tissue culture level do not always predict the in vivo results. Even if certain compounds seemed to be strongly effective, direct use of such potentially risky

drugs for human use should be avoided from an ethical viewpoint. Therefore, at least the safety and efficacy of those compounds should be tested in the animal model.

However, there is a big hindrance to conduct the animal experiment in the AIDS research. HIV-1 can only infect a limited species of nonhuman primate: chimpanzees. Experiments using chimpanzees are nowadays very difficult because it is an endangered species and therefore listed in the red data booked animal. In addition, it is not a good model because chimpanzees hardly induce AIDS-like symptoms even the infection was successfully attained (Novembre et al., 1997). Under these circumstances such conditions, simian immunodeficiency virus (SIV) that is the closely related virus to HIV-1 has been closed up. The discovery of HIV-1 as the causative virus of AIDS by Barre-Sinoussi et al. (1983) was followed by the discoveries of SIVs from various species of monkeys. Among them, SIVmac which was initially reported to be isolated from rhesus macaques have been used to study the viral pathogenicity using macaques because this species is rather easily available for researchers. It is already known that the macaque monkeys infected with SIVmac develop simian acquired immunodeficiency syndrome (SAIDS) within a few years post infection and the

symptoms are very similar to human AIDS (Letvin et al., 1985). Thereafter, the SIV/monkey model system has been widely utilized for the studies in vaccine development and elucidation for the viral pathogenesis.

Since the clinical success of HAART is great, one can naturally think why we do not conduct further tests of new drugs using the SIVmac/monkey model. However, here is another big barrier. Some of the anti-HIV-1 drugs do not affect the replication of SHIV to the same extent to HIV-1. It is considered that a slight difference between HIV-1 proteins and SIV proteins at the 3D structural level could be a reason for this. Present anti-HIV drugs are specifically designed targeting the HIV-1 proteins. Let's take an example of inhibitors toward reverse transcriptase (RT). RT is the first one of the targets that were documented to develop anti-retroviral drugs because the enzyme is a viral genome-encoded protein and the virus cannot replicate without its normal function.

Azidothymidine (AZT) is the firstly used compound as the anti-HIV drug. The inhibitors targeting RT are nowadays classified into two groups: nucleotide RT inhibitors (NRTIs) and non-nucleotide RT inhibitors (NNRTIs). AZT is a representative

drug of the former group. Since the former group mimic the substrate for reverse transcriptase (RNA dependent DNA polymerase), the drugs of this group bind to the catalytic site of both HIV-1 and SIV, and therefore they work on both types. On the other hand, NNRTIs bind to the RT molecules in a type-specific manner. This means that most NNRTIs do not block the replication of SIV at all.

To overcome the above-mentioned dilemma, Überla et al. (1995) constructed a new SIV/HIV chimeric virus termed RT-SHIV in which the RT gene is derived from HIV-1. This virus could infect and replicate in monkeys. This virus also showed almost the equal sensitivity toward an anti-HIV-1 drug called nevirapine, a typical NNRTI, in a type-specific manner not only *in vitro* but also *in vivo*. Currently the RT-SHIV/monkey model is thought to be useful for the evaluation of NNRTIs, and such a pilot study using RT-SHIV was recently reported by North et al. (2005).

As the anti-HIV drugs, another posssible target is the viral protease (PR). (Recently the viral integrase (INT) is added to the list of promising target molecules.) PR is a small viral protein consisting of 99 amino acid (AA) residues (297 bases). Two molecules of PR are known to form a dimmer and to carry out auto processing of Gag

and Pol polyproteins at several cleavage sites, which is a necessary steps for the assembly showing maturation of viral component proteins. HIV and SIV are genetically closely related and approximately 70 % homology in the AA level. Most of the PR inhibitors are peptide-based, substrate analogues. They occupy a narrow catalytic pocket that is formed by the interface of two monomers and block further processing of substrate polyproteins. But, probably due to a narrow structural stringency, PR inhibitors that were developed specifically against HIV-1 do not effectively work on the PR of SIV.

Under these circumstances, we have generated a novel SHIV-pr in which the PR gene alone is derived from HIV-1. Newly constructed SHIV-pr could replicate in monkey PBMC *in vitro*. Then, we conducted monkey infection experiments. SHIV-pr was intravenously inoculated into two rhesus macaques. One monkey resulted in a weak but rather long lasting persistent infection whereas the infection of another was only temporary. Then we performed the *in vivo* passage up to the forth generation. This passage conferred a strong productivity to the virus and eventually resulted in a robust persistent infection that is almost comparable to a parental virus, SIVmac. To examine

whether the SHIV-pr/monkey system can be used for evaluating PR inhibitors, the contents of a Kaletra capsule (a mixture of ritonavir [Sham et al., 1998] and lopinavir) was orally administered to three SHIV-pr infected monkeys for 4 weeks. The results obtained in the present study indicated that SHIV-pr could be used for *in vivo* efficacy tests towards anti-HIV-1 PR inhibitors using monkeys.

MATERIALS & METHODS

DNA constructs

Infectious molecular clones of HIV-1 (pNL432) (Adachi et al., 1986) and SIVmac239 (pMA239) (Shibata et al., 1991) were used as parent proviral DNAs.in this study. The genomic organization of SHIV-pr, in which the PR gene of SIVmac was replaced by that of HIV-1, is shown in Fig. 1A, and its more detailed structure is shown in Fig. 1B. Chimeric junctions were generated at the N-terminal and C-terminal ends of the viral PR gene by polymerase chain reaction (PCR)-based site-directed mutagenesis as follows. First, the *Spe I* (nt 1507)-*Sse 8387I* (nt 2839) gene fragment of pNL432 and the *Spe I* (nt 2026)-*Sse 8387I* (nt 3397) gene fragment of pMA239, both of which span the region from the middle of the capside protein (CA) gene to the N-terminal region of reverse transcriptase (RT) gene, were cut out by restriction enzymes and then inserted into pUCTA119 (Ido and Hayami, 1997), which was modified from pUC119 to possess a *Spe I* site at the multiple cloning site and precut by *Spe I* and *Pst I*. The viral gene-inserted plasmids were named pHIV-CAPR and pSIV-CAPR respectively. Then PCR

was run using a primer SHPR-F 5'-TTAGTGCACCTCAGATCACTCTTTGGCAGCGACCCC-3' and an M13 forward primer and pHIV-CAPR as a template. Digestion of this PCR product with *Bsp 1286I* (*Bsp 1286I* site underlined) and *Sse 8387I* yielded a fragment containing the HIV-1 PR genomic region with a sticky ACGT overhang at the N-terminal side of the PR gene. Another PCR was run using a primer SHPR-R 5'-TTGAGCTGCAGCAAATCCTCTGTCACCTCC-3' and an M13 reverse primer and pSIV-CAPR as a template. Digestion of this PCR product with *Spe I* and *Pst I* (*Pst I* site underlined) yielded a fragment containing the 3' half of the SIVmac *gag* gene with a sticky TGCA overhang near the C-terminal side of *gag* gene. The two fragments obtained were ligated and subcloned into pUCTA119 precut with *Spe I* and *Pst I*. By doing so, a SIVmac/HIV-1 chimeric junction was made at the N-terminus of PR gene. To generate a chimeric junction at the C-terminus of the PR gene, we first introduced a *Dra I* site near the C-terminus of the PR gene of pSIV-CAPR because pNL432 (HIV-1) already possesses the same site exactly at the corresponding position (nt 2540) and creating a *Dra I* made it easy to produce the chimeric junction. This mutation itself does

not alter the amino acid sequence of PR. PCR was run using a primer MAPR-R 5'-CTTTAGCTA TGGGAAAATTTAAAGACATCCCCAGAGCTG-3' (*Dra I* site underlined) and an M13 reverse primer and pSIV-CAPR as a template. After creating a *Dra I* site, an HIV-1/SIVmac chimeric junction at the C-terminus of the PR region was made by conventional molecular techniques. Finally, the *Spe I-Sse 8387I* fragment having the HIV-1 PR gene in a SIVmac background was transferred to the corresponding position of pMA239. This molecular clone of a novel SHIV having the HIV-1 PR gene in the SIVmac genome was termed pSHIV-pr. A *Dra I*-incorporated mutant full-genome plasmid of pMA239 was also made and named pSIVmac-mD.

Cell culture

M8166 is a subclone of C8166 (Clapham et al., 1987), a $CD4^+$ human T-cell line. HSC-F is a cynomolgous monkey $CD4^+$ T-cell line from a fet al splenocyte that was immortalized by infection with *Herpesvirus saimiri* subtype C (Akari et al., 1996). M8166 cells and HSC-F cells were maintained in RPMI 1640 medium containing 10 % heat-inactivated fet al bovine serum (FBS). PBMCs of healthy rhesus monkeys were

separated from heparinized whole blood by Percoll density gradient centrifugation, stimulated with 25 mg/ml of concanavalin A for 24 h, and maintained in RPMI 1640 medium containing 10 % FBS and 400 unit/ml of recombinant human interleukin 2 (IL-2) as described previously (Kuwata et al., 1995).

Transfection and infection

To generate infectious virus particles from a full genome plasmid DNA, typically 5 μg of pSHIV-pr was introduced into 1.5×10^6 M8166 cells by the DEAE-dextran method (Naidu et al., 1988). The culture supernatants were harvested every three days and stored at -80 ℃. Then, virion-associated RT activity was measured as described previously (Willey et al., 1988). The fractions with the highest RT activities were pooled, filtered (0.45 μm pore size) and stored as a virus stock at – 80 ℃. To determine the viral infectivity of the virus stock, the 50 % tissue culture infectious dose ($TCID_{50}$) was calculated by using M8166 cells. The virus inoculum used for *in vitro* infection was adjusted to contain $3\text{-}4\times10^3$ RT units by adding the appropriate volume of the medium to the virus stock. M8166 cells, HSC-F cells or monkey PBMCs (5×10^4 cells/well)

were infected with a virus and cultured in a 96-well plate. The culture supernatants were harvested every 3 days and production of the virions was monitored by measuring their RT activities.

Western blotting

Expression of the viral proteins after transfection by the DEAE-dextran method was examined by Western immunoblotting. M8166 cells were transfected with proviral plasmids and the cell lysates were prepared, resolved on 12 % polyacrylamide gels and Gag precursor polyproteins and its protease-cleaved products were detected by Western immunoblotting using a SHIV-3rN-inoculated antiserum (Kuwata et al., 1995). SHIV-3rN-inoculated monkeys developed strong high titers of antibodies against the viral component proteins especially Gag and Pol proteins. Antiserum of these monkeys was used to detect the SIVmac-derived viral structural proteins.

Effect of KNI-272, a PR inhibitor, on viral replication

KNI-272, a peptide-analog PR inhibitor (Doi et al., 2001), was kindly provided by

Prof. Yoshiaki Kiso, Department of Medical Chemistry, Kyoto Pharmaceutical College, Kyoto, Japan.

Inoculation of macaques and *in vivo* passage

Six rhesus macaques (*Macaca mulatta*) negative for SIV and simian T-cell lymphotropic virus were used in the present study. All the monkeys were intravenously inoculated with SHIV-pr through the foot saphenous vein. First we inoculated a cell-free virus stock of SHIV-pr (2×10^5 $TCID_{50}$) to two rhesus macaques (MM236 and MM239 [1st]). For the second generation, we injected plasma taken from MM239 at 2, 27, and 51 weeks post infection (w.p.i.) into MM275 (A flowchart of *in vivo* passages is given in Fig. 4C). For the third generation we injected plasma and minced lymph nodes from MM275 at 17 days post infection into MM274. For the fourth generation, we injected a mixture of plasma and isolated virus from PBMCs of MM274 at 3 w.p.i. into MM289 and MM294. All animals were housed in a P3-level monkey storage facility, and were treated in accordance with regulations approved by the Committee for Experimental Use of Nonhuman Primates in the Institute for Virus Research, Kyoto University.

Flow cytometry

The CD4+ and CD8+ T lymphocyte subset in PBMC were examined by flow cytometry. Whole blood samples of the monkeys were stained with phycoerythrin (PE) - conjugated anti-CD4 monoclonal antibody (NU-TH/I; Nichirei, Japan) and PerCP-conjugated anti-CD8 monoclonal antibody (BD PharMingen). After hemolysis of the blood samples using lysing solution (BD PharMingen), there were analyzed on a FACScan (Becton-Dickinson, Mountain View, CA).

Virus isolation

We attempted to isolate infectious viruses from the PBMCs of inoculated monkeys as follows. PBMCs (typically 1x 10^6 cells) were cocultured with 1x10^6 M8166 cells for at least 4 weeks in RPMI 1640 containing 10 % of FBS in a 24-well plate. Virus recovery was judged by the syncytial cytopathic effect (CPE) by a microscopic observation and a rise of RT activity of the culture supernatants.

Detection of plasma viral RNA

Plasma viral RNA loads were determined by a Taqman RT-PCR kits (Perkin-Elmer, New Jersey, USA) using the SIV gag region primers SIVII-696F (5' GGAAATTACCCAGTACAACAAATAGG 3') and SIVII-784R (5' TCTATCAATTTTACCCAAGGCATTTA 3') and SIVII-731T (5' Fam-TGTCCACCTGCCATTAAGCCCG-Tamra 3') as a labelled probe that was used for the detection of PCR products. For each run, a standard curve was generated from dilutions of a standard sample, measured by the Branched DNA method (Bayer Diagnostics).

Titration of antibody

Antibody of each generated monkeys plasmas after inoculation with SHIV-pr were titrated by particle agglutination according to the instructions of the Serodia HIV-1/2 kit (Fujirebio, Japan).

Sequencing analyses

For the identification of the mutations in the SHIV-pr genomic region of PR

and Gag that occurred during the construction, the *in vivo* passage (3weeks from MM275 [2nd] post infection, 1 weeks post infection from MM274 [3rd]) and 2 weeks post infection from MM289 [4th]) or protease inhibitor treatment (before [0day] and after [1 weeks post medication] treatment from MM289 and MM275), sequencing analyses are performed. Virion RNA was extracted from plasma samples using a Viral RNA kit (QIAGEN). We also performed sequencing analyses by RT-PCR of the extracted RNAs and PCR using the primer pair SIV7F (5' GGAAATGTGGAAAAATGGACC 3') and SIV9.5R (5' GCATTCTCCATTTGTTCTTATCC 3').

Protease inhibitor treatment *in vivo*

The contents of a Kaletra capsule (a mixture of lopinavir/ritonavir, Abbot, Japan) was suspended in drinking water and administered by the oral route (12 mg of lopinavir and 3 mg of ritonavir/kg/day) to 3 monkeys (MM275, MM289 and MM294) for 4 weeks.

RESULTS

Construction of SHIV-pr having the HIV-1-derived PR genomic region

The genomic organization ofSHIV-pr, is shown in Fig. 1A, and its more detailed structure is shown in Fig. 1B. To generate this noble chimeric virus we first introduced a *Dra I* site into the viral genome of SIVmac239. Introduction of this site made it easy to produce a chimeric junction near the C-terminal end of the PR gene since a molecular clone of HIV-1 (pNL432) already possesses the same site exactly at the corresponding position. This mutation itself does not alter amino acid sequences of PR and this mutant virus was named SIVmac-mD. Constructing a chimeric junction at the N-terminal end of PR gene needed a bit tricky strategy. We paid attention to a 4-letter-palindromic sequence TGCA of SIVmac immediately before the N-teriminal proline codon of PR. Ligation of two sticky overhangs produced by digestion of the PCR products by *Bsp 1286I* and *Pst I* made it possible as described in Materials and methods. The final full-genome plasmid clone of SHIV-pr was named pSHIV-pr, and a Dra I-incorporated mutant full-genome clone of SIVmac 239 was named pSIVmac-mD.

Autoprocessing of Gag proteins of SHIV-pr

To clarify whether the HIV-1-derived PR could undergo authentic proteolytic processing of Gag precursor polyproteins, we examined lysates of virus-infected cells with Western blot. As shown in Fig. 2, autoproccessing patterns of p55 Gag precursor proteins of SHIV-pr, SIVmac, and SIVmac-mD were basically equal and bands of p26 capsid and p17 matrix proteins as well as p41 partially processed intermediate products were clearly detected, suggesting that the PR of SHIV-pr can recognize and cut the cleavage sites although its proteolytic activity seems to be slightly lower as judged by the band intensities.

Replication of SHIV-pr at the *in vitro* tissue culture level

The growth-competence of SHIV-pr as well as its those of parental viruses HIV-1 and SIVmac were examined in human and monkey lymphoid cell lines, M8166 and HSC-F, respectively (Fig. 3). As shown in Fig. 3A, SHIV-pr replicated fairly well, with showing conspicuous ballooning in M8166 cells although the start and peak of

viral growth were delayed several days compared with those of SIVmac. SHIV-pr was also replication-competent in a cynomolgous monkey-derived cell line, HSC-F cells, whereas HIV-1 could not replicate at all (Fig. 3B). In this monkey cell line, the growth of SHIV-pr was obviously delayed compared with that of SIVmac, but more importantly SHIV-pr exhibited an ability to replicate in monkey cells.

Effect of a PR inhibitor, KNI-272, on the viral growth of SHIV-pr and SIVmac in HSC-F cells and macaque PBMCs

To clarify whether a PR inhibitor, KNI-272, is effective on the growth of SHIV-pr and its parental SIVmac or not, the growth-kinetics in HSC-F cells and lowermacaque PBMCs were examined in the presence and absence of the inhibitor. KNI-272 is a tripeptide-like protease inhibitor which mimics the amino acid sequence of the cleavage site between p17 matrix and p24 capsid proteins in Gag precursor proteins. The replication of SIVmac was slightly inhibited by KNI-272 at a concentration of 100 nM and blocked at a concentration of 1000 nM in HSC-F cells (Fig. 3C). On the other hand, the growth of SHIV-pr was completely inhibited by the inhibitor even at a concentration

of 100 nM in both HSC-F cells and macaque PBMCs (Fig. 3C and Fig. 3D respectively).

Infection of SHIV-pr to macaque monkeys

Since SHIV-pr was found to be replication-competent in monkey PBMCs, we then inoculated the virus into two macaques (MM236 and MM239). The results are summarized in Table 1. The virus was reisolated from both monkeys. MM239 showed strong induction of antibodies judged by PA tests while MM236 showed only a slight rise of PA titer. The plasma viral RNA loads of both monkeys were within a range of 10^3-10^4 copies/ml from 2 –12 weeks post infection (see Fig. 4A for the data of MM239), but thereafter decreased below the detection limit (500 copies/ml) except that the virus was reisolated from MM239 alone and the RNA load was 10^4 copies/ml at 27 weeks post infection. MM239 died at 51 weeks. However, post-mortem examination did not reveal any signs of AIDS-like symptoms except a slight trait of pneumonia in the lungs, and the CD4 cell counts remained within the normal range. Overall, the inoculation of SHIV-pr seemed to have resulted in a rather weak systemic infection.

***In vivo* passage**

Since SHIV-pr did not productively replicate in the inoculated monkeys (1st generation), we conducted four *in vivo* passages to enhance viral productivity. The results are summarized in Fig. 4 (A: plasma viral RNA loads; B: CD4 cell counts; C: a flowchart of passages). As shown in Fig. 4A, SHIV-pr gradually enhanced its viral productivity and the peak values of viral RNA loads reached 10^5 copies/ml in the 2nd generation. In the 3rd (MM274) and 4th (MM289 and MM294) generations, it reached the values of 10^6 or 10^7 copies/ml with rather high set points (around 10^5 copies/ml) which are comparable to those of SIVmac. At 4-6 weeks post infection, $CD4^+$ cells of four of five monkeys decreased to approximately the 50 % level but gradually recovered to the normal level (Fig. 4B).

Mutations in the PR domain during the course of *in vivo* passages

We considered that the virus SHIV-pr might have adapted to rhesus macaques because its productivity had been increased generation by generation during the course of *in vivo* passages. It was likely that the mutations might have occurred in the PR

genomic region, because only the PR is derived from HIV-1. Therefore, we analyzed the sequence of the PR gene of each generation using plasmas taken at the early time of infection (1 w. p. i. [MM274], 2w. p. i. [MM289] or 3w. p. i. [MM275]) by RT-PCR. We found two nucleotide mutations in the PR region (Table 2A): C219T and G229A in the viral RNAs extracted from the plasmas of the 2nd (MM275), 3rd (MM274) and 4th (MM289) generations of SHIV-pr-infected monkeys. These two mutations were commonly found in these three monkeys. Both nucleotide changes lead to amino acid changes, A71V and V77I, respectively (Table 2B). These two changes were not the reversion to the amino acid residues of SIVmac. Gag precursor polyproteins were cleaved at several cleavage sites by the viral PR to yield mature Gag proteins MA, CA, NC and p6 (Fig. 6) (Henderson et al., 1988) . We found 4 common mutations in the Gag region in the 2nd to 4th generations (Table 3): R276K, V375M, D429N and G433D. G433D is at the C-terminal end of NC and close to the cleavage site between NC and p1.

Administration of Kaletra by the oral route to SHIV-pr-infected monkeys

Whether SHIV-pr-infected monkeys respond to protease inhibitors or not critically determines the value of this monkey model system. Now that we had monkeys with steady set points (MM289, MM294, and MM275), we examined the effect of the PR inhibitor mixture Kaletra on their viral loads and $CD4^+$ counts.

The effect of Kaletra treatment was dramatic, causing the plasma viral RNA loads of all three monkeys to rapidly decrease to near or below the detection limit (Fig. 5A). However, the viral loads quickly rebounded to the levels after the cessation of drug administration. Especially in MM289, the load rebounded to the pre-treatment level within one week and maintained its high value thereafter. In other two monkeys, the loads also rebounded but they did not reach the pre-treatment levels. The numbers of $CD4^+$ cells did not change during the medication in any of the monkeys. However, from the second week after the cessation of medication, the number of $CD4^+$ cells in MM294 showed a tendency to recover in MM294. No significant changes of $CD4^+$ counts were observed in MM275 or MM289 (Fig. 5B).

Comparison of mutations in the PR genomic region before and after the Kaletra treatment

Whenever we do a medication using anti-viral drugs, we need to take heed on the emergence of escape mutants. Kaletra is one of the commercially available PR inhibitors. We checked whether there were any mutations in the sequences of viral RNA or not extracted from the plasmas MM275 and MM289 before (0day) and 1 week after the medication. We found many nucleotide mutations in the PR region of viral genes extracted from MM275 and MM289 at the time of pre-medication. It is no wonder because the monkeys had been inoculated with SHIV-pr for long periods about 110 or 70 weeks respectively. However, we detected only one mutation in the PR region when we compared the sequences before and after the Kaletra treatment (Table 4). In MM289, we detected no mutational changes before and after the medication. We also examined the Gag region, because it was reported that mutations near the cleavage sites in the Gag region conferred high resistance against protease inhibitors. However, we detected only one mutation M474I on the Gag region, which is rather distant from the cleavage site (data not shown).

DISCUSSION

Animal model for AIDS is difficult because HIV-1 can only infect humans and limited species of nonhuman primates. The SIV/monkey model have been widely used for AIDS research. To evaluate anti-HIV drugs, however we confront a difficult generation because the drugs are usually specifically designed for HIV-1 and their effect on SIV is not the same as that on HIV-1. A SHIV termed RT-SHIV that possesses RT of HIV-1 was generated by Überla et al. (1995) and nowadays used for evaluation of NRRTIs that specifically inhibit HIV-1 (Hofman et al., 2004 and Balzarini et al., 1995). Other than RT-SHIV, there was no documented report of SHIVs that have HIV-1-derive gene in the *pol* region, to the best of the author's knowledge.

In the present study we constructed a SHIV having the PR of HIV-1 in the SIVmac genome. The generated SHIV-pr was replication-competent in not only a human cell line but also a monkey cell line. The virus could also replicate even in monkeys. This is the first report of a SHIV with the PR gene of HIV-1 that is growth-competent in macaques. As revealed in Western immunoblotting, the replacement of PR

did not significantly affect the autoproccessing of Gag polyproteins, suggesting that HIV-1 PR can recognize the same cleavage sites of SIVmac polyproteins as SIVmac PR. However, the growth-potential of SHIV-pr seemed to be less than that of the parental virus SIVmac based on the delay of the rise of RT activities and a lower peak value in the growth-kinetic profiles. The slightly lower activity of PR of SHIV-pr observed in Fig. 2 could partly contribute the delayed virus growth during propagation *in vitro* (perhaps *in vivo* too) observed in Fig. 3. Further studies are needed to understand the mechanism of this weakening of viral productivity.

Inoculation of SHIV-pr to macaque monkeys resulted in a rather weak systemic infection. The replacement of PR might have disrupted the smooth maturation of viral structural proteins and consequently weakened the viral replication ability. Nonetheless, the *in vivo* passage clearly conferred a robust productivity to the virus. We are currently attempting to identify the mutations responsible for the increased productivity. A molecular clone of the monkey-adapted virus should also be pursued as the next step.

We found two mutations in the region of PR: C219T and G229A from the plasmas taken from 2nd (MM275), 3rd (MM274) and 4th (MM289) SHIV-pr-infected

monkeys. These two mutations were commonly found in all the three monkeys. Both mutations resulted in amino acid changes, A71V and V77I respectively. These two amino acid changes are considered to have occurred as a result of adaptation to monkeys. However, these were not the residues of SIVmac. On the other hand, we found 4 common mutations in the Gag region in the 2nd to 4th generations. G433D is at the C-terminal end of NC and close to the cleavage site between NC and p1, and D429N is also near this cleavage site. Gag proteins are cleaved at several cleavage sites by the viral PR. Therefore, this mutation might have played a critical role to enhance the viral productivity of SHIV-pr. We also found some minor mutations (30 % or less in the peak height) in the Gag region especially in the specimens of the 3rd and 4th (data not shown). Although the viruses of the 3rd and 4th generations showed strong viral productivity compared with that of the 2nd generation, we did not find any significant mutational changes between the 2nd and 3rd or 4th generations. Thus, in order to determine the mutations that are responsible for enhancement of viral productivity, we probably need to consider the significances of such minor mutations in other genomic regions.

Our main finding is that SHIV-pr could replicate in macaques and was sensitive to protease inhibitors. Our results demonstrate that the SHIV-pr/monkey system can be used for *in vivo* efficacy tests of protease inhibitors.

As an example of a SHIV that has chimeric boundaries in either *gag* or *pol* genomic region, RT-SHIV (Überla et al., 1995) is already known. RT-SHIV possesses the HIV-1-derived RT gene and it could replicate in macaques. In addition, RT-SHIV was almost as sensitive to NNRTIs *in vivo* as it was *in vitro* (Balzarini et al., 1995). In fact, the virus was used to evaluate NNRTIs (North et al., 2005 and Hofman et al., 2004). Because SHIV-pr can be used for a similar purpose, it would be interesting to generate a new SHIV that possesses the whole *pol* genomic region of HIV-1 and to use it to conduct experiments of the HAART model.

In an *in vitro* study, it was reported that combination of lopinavir and ritonavir yielded escape mutants in HIV-1 such as I84V or I50V/M46I (Johnson et. el., 2004). We detected only one mutation L33V in MM275 in the PR region when compared before and after the Kaletra treatment. The mutation L33F was reported as one of the eight mutations in the PR region found in the resistant virus against Kaletra (Mo et. el., 2003).

Whether the mutation of L33V alone is sufficient to show resistance against Kaletra or not is unknown. It is also said that mutations caused by PR inhibitors often appear in the Gag region especially near the each cleavage sites (La Seta Catamancio et al., 2001) In the Gag region, we detected only one mutation M474I, which is not near the cleavage site. We did not observe any mutations during the PR inhibitor treatment, indicating Kaletra was quite effective without producing any escape mutants at least for 4 weeks. On a clinical trial, it was reported that isolated virus obtained from patients who underwent a medication by the Kaletra treatment showed a strong sensitiveness to the drug over 12 weeks (according to the appendex of Kaletra). The active site of PR is at the central cavity of the dimmer formation. The position of L33V is a little distant from the cavity in the 3D structure model (Hoog et al., 1996). Although clinical data of appearance of escape mutants by use of some PR inhibitors including ritonavir were reported (Zennou et al., 1998), there were no common mutations compared with those observed in this study.

Two groups recently developed viruses whose genomes are mostly derived from HIV-1 (Kamada et al., 2006 and Hatziioannou et al., 2006). APOBEC3 family proteins

and TRIM5 α have recently been identified as molecules that determine species tropism. These findings may help to create viruses that differ from the HIV-1 genome by only one or two genes, such as the capsid and *vif* genes. These viruses can replicate in monkey-derived cells and possibly in monkey PBMCs. However, to the best of our knowledge, the viruses cannot replicate in rhesus macaques. Because SHIV-pr can replicate in rhesus macaques with steady and rather high values of setpoints, it appears to be best suited for evaluating HIV-1-targeted PR inhibitors in monkeys.

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REFERENCE

Adachi, A., H. E. Gendelman, S. Koenig, T. Folks, R. Willey, A. Rabson, and M. A. Martin. 1986. Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone. J Virol 59:284-91.

Akari, H., K. Mori, K. Terao, I. Otani, M. Fukasawa, R. Mukai, and Y. Yoshikawa. 1996. *In vitro* immortalization of Old World monkey T lymphocytes with Herpesvirus saimiri: its susceptibility to infection with simian immunodeficiency viruses. Virology 218:382-8.

Balzarini, J., M. Weeger, M. J. Camarasa, E. De Clercq, and K. Überla. 1995. Sensitivity/resistance profile of a simian immunodeficiency virus containing the reverse transcriptase gene of human immunodeficiency virus type 1 (HIV-1) toward the HIV-1-specific non-nucleoside reverse transcriptase inhibitors. Biochem Biophys Res Commun

211:850-6.

Barre-Sinoussi, F., J. C. Chermann, F. Rey, M. T. Nugeyre, S. Chamaret, J. Gruest, C. Dauguet, C. Axler-Blin, F. Vezinet-Brun, C. Rouzioux, W. Rozenbaum, and L. Montagnier. 1983. Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). Science 220:868-71.

Clapham, P. R., R. A. Weiss, A. G. Dalgleish, M. Exley, D. Whitby, and N. Hogg. 1987. Human immunodeficiency virus infection of monocytic and T-lymphocytic cells: receptor modulation and differentiation induced by phorbol ester. Virology 158:44-51.

Colson, P., M. Henry, C. Tourres, D. Lozachmeur, H. Gallais, J. A. Gastaut, J. Moreau, and C. Tamalet. 2004. Polymorphism and drug-selected mutations in the protease gene of human immunodeficiency virus type 2 from patients living in Southern France. J Clin Microbiol 42:570-7.

Doi, M., T. Ishida, Y. Katsuya, M. Sasaki, T. Taniguchi, H. Hasegawa, T. Mimoto, and Y. Kiso. 2001. KNI-272, a highly selective and potent peptidic HIV protease inhibitor. Acta Crystallogr C 57:1333-5.

Hatziioannou, T., M. Princiotta, M. Piatak, Jr., F. Yuan, F. Zhang, J. D. Lifson, and P. D. Bieniasz. 2006. Generation of simian-tropic HIV-1 by restriction factor evasion. Science 314:95.

Henderson, L. E., R. E. Benveniste, R. Sowder, T. D. Copeland, A. M. Schultz, and S. Oroszlan. 1988. Molecular characterization of gag proteins from simian immunodeficiency virus (SIVMne). J Virol 62:2587-95.

Hofman, M. J., J. Higgins, T. B. Matthews, N. C. Pedersen, C. Tan, R. F. Schinazi, and T. W. North. 2004. Efavirenz therapy in rhesus macaques infected with a chimera of simian immunodeficiency virus containing reverse transcriptase from human immunodeficiency virus type 1. Antimicrob Agents Chemother 48:3483-90.

Hoog, S. S., E. M. Towler, B. Zhao, M. L. Doyle, C. Debouck, and S. S. Abdel-Meguid. 1996. Human immunodeficiency virus protease ligand specificity conferred by residues outside of the active site cavity. Biochemistry 35:10279-86.

Ido, E., and M. Hayami. 1997. Construction of T-tailed vectors derived from a pUC plasmid: a rapid system for direct cloning of unmodified PCR products. Biosci Biotechnol Biochem 61:1766-7.

Johnson, V. A., F. Brun-Vezinet, B. Clotet, B. Conway, R. T. D'Aquila, L. M. Demeter, D. R. Kuritzkes, D. Pillay, J. M. Schapiro, A. Telenti, and D. D. Richman. 2004. Update of the drug resistance mutations in HIV-1: 2004. Top HIV Med 12:119-24.

Kamada, K., T. Igarashi, M. A. Martin, B. Khamsri, K. Hatcho, T. Yamashita, M. Fujita, T. Uchiyama, and A. Adachi. 2006. Generation of HIV-1 derivatives that productively infect macaque monkey lymphoid cells. Proc Natl Acad Sci U S A 103:16959-64.

Kuwata, T., T. Igarashi, E. Ido, M. Jin, A. Mizuno, J. Chen, and M. Hayami. 1995. Construction of human immunodeficiency virus 1/simian immunodeficiency virus strain mac chimeric viruses having vpr and/or nef of different parental origins and their *in vitro* and *in vivo* replication. J Gen Virol 76 (Pt 9):2181-91.

La Seta Catamancio, S., M. P. De Pasquale, P. Citterio, S. Kurtagic, M. Galli, and S. Rusconi. 2001. *In vitro* evolution of the human immunodeficiency virus type 1 gag-protease region and maintenance of reverse transcriptase resistance following prolonged drug exposure. J Clin Microbiol 39:1124-9.

Letvin, N. L., M. D. Daniel, P. K. Sehgal, R. C. Desrosiers, R. D. Hunt, L. M. Waldron, J. J. MacKey, D. K. Schmidt, L. V. Chalifoux, and N. W. King. 1985. Induction of AIDS-like disease in macaque monkeys with T-cell tropic retrovirus STLV-III. Science 230:71-3.

Mo, H., L. Lu, T. Dekhtyar, K. D. Stewart, E. Sun, D. J. Kempf, and A. Molla. 2003.

Characterization of resistant HIV variants generated by *in vitro* passage with lopinavir/ritonavir. Antiviral Res 59:173-80.

Naidu, Y. M., H. W. Kestler, 3rd, Y. Li, C. V. Butler, D. P. Silva, D. K. Schmidt, C. D. Troup, P. K. Sehgal, P. Sonigo, M. D. Daniel, and et al. 1988. Characterization of infectious molecular clones of simian immunodeficiency virus (SIVmac) and human immunodeficiency virus type 2: persistent infection of rhesus monkeys with molecularly cloned SIVmac. J Virol 62:4691-6.

North, T. W., K. K. Van Rompay, J. Higgins, T. B. Matthews, D. A. Wadford, N. C. Pedersen, and R. F. Schinazi. 2005. Suppression of virus load by highly active antiretroviral therapy in rhesus macaques infected with a recombinant simian immunodeficiency virus containing reverse transcriptase from human immunodeficiency virus type 1. J Virol 79:7349-54.

Novembre, F. J., M. Saucier, D. C. Anderson, S. A. Klumpp, S. P. O'Neil, C. R. Brown,

2nd, C. E. Hart, P. C. Guenthner, R. B. Swenson, and H. M. McClure. 1997. Development of AIDS in a chimpanzee infected with human immunodeficiency virus type 1. J Virol 71:4086-91.

Sham, H. L., D. J. Kempf, A. Molla, K. C. Marsh, G. N. Kumar, C. M. Chen, W. Kati, K. Stewart, R. Lal, A. Hsu, D. Betebenner, M. Korneyeva, S. Vasavanonda, E. McDonald, A. Saldivar, N. Wideburg, X. Chen, P. Niu, C. Park, V. Jayanti, B. Grabowski, G. R. Granneman, E. Sun, A. J. Japour, J. M. Leonard, J. J. Plattner, and D. W. Norbeck. 1998. ABT-378, a highly potent inhibitor of the human immunodeficiency virus protease. Antimicrob Agents Chemother 42:3218-24.

Shibata, R., M. Kawamura, H. Sakai, M. Hayami, A. Ishimoto, and A. Adachi. 1991. Generation of a chimeric human and simian immunodeficiency virus infectious to monkey peripheral blood mononuclear cells. J Virol 65:3514-20.

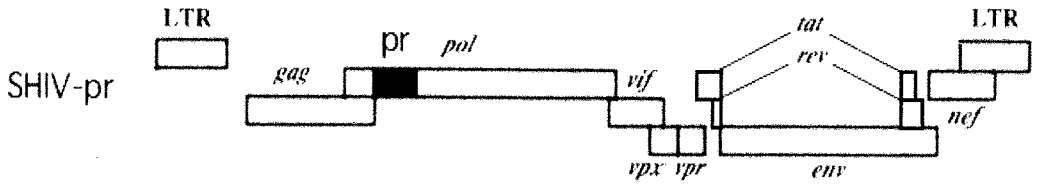
Überla, K., C. Stahl-Hennig, D. Bottiger, K. Matz-Rensing, F. J. Kaup, J. Li, W. A.

Haseltine, B. Fleckenstein, G. Hunsmann, B. Oberg, and et al. 1995. Animal model for the therapy of acquired immunodeficiency syndrome with reverse transcriptase inhibitors. Proc Natl Acad Sci U S A 92:8210-4.

Willey, R. L., D. H. Smith, L. A. Lasky, T. S. Theodore, P. L. Earl, B. Moss, D. J. Capon, and M. A. Martin. 1988. *In vitro* mutagenesis identifies a region within the envelope gene of the human immunodeficiency virus that is critical for infectivity. J Virol 62:139-47.

Zennou, V., F. Mammano, S. Paulous, D. Mathez, and F. Clavel. 1998. Loss of viral fitness associated with multiple Gag and Gag-Pol processing defects in human immunodeficiency virus type 1 variants selected for resistance to protease inhibitors *in vivo*. J Virol 72:3300-6.

(A)



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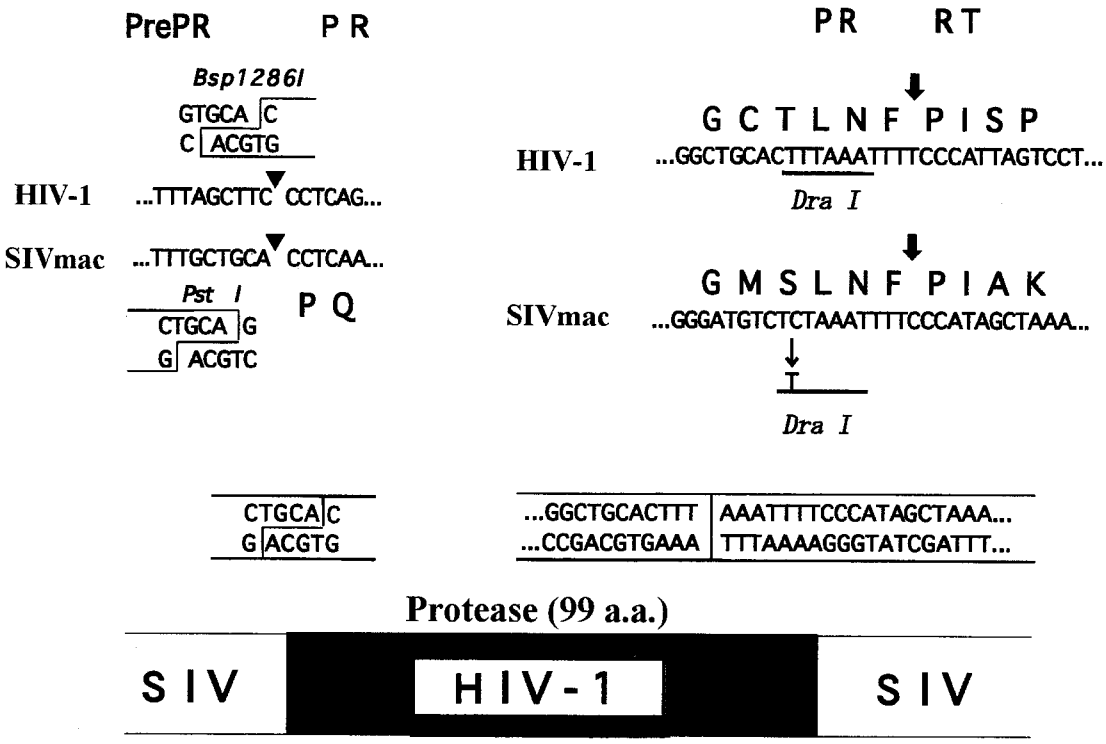

Fig. 1 Construction of SHIV-pr having the HIV-1 PR gene in the SIVmac genome. A) Genomic organization of SHIV-pr. The HIV-1-derived genomic region is indicated by a black bar. B) Nucleotide sequences near the N-terminal and C-terminal ends of PR genes of HIV-1 and SIVmac. The arrows indicate the cleavage sites by viral protease. The detailed procedures of construction of chimeric junctions in SHIV-pr are described in Materials and methods.

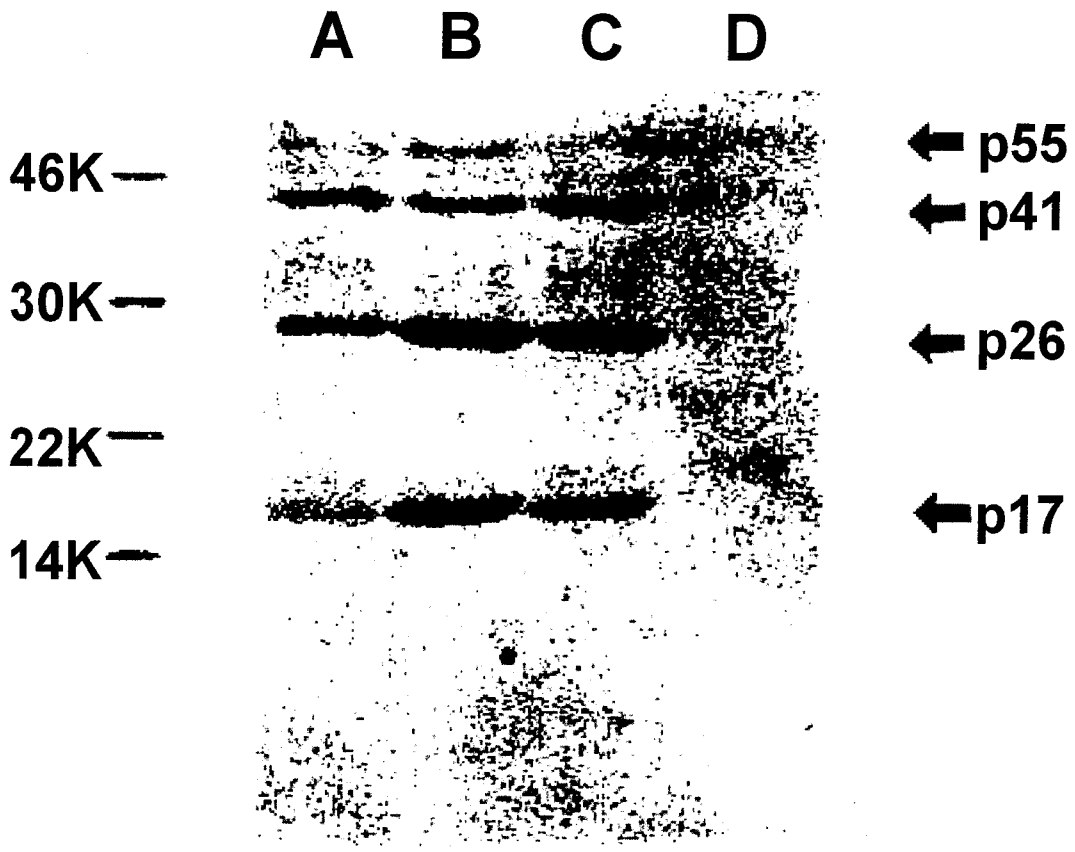

Fig. 2 Immunoblotting analysis of proteolytic processing of Gag proteins. The lysates of M8166 cells transfected with plasmids of a chimeric and a parental virus were harvested at 48 hr and resolved on a 12 % SDS-polyacrylamide gel. Immunoblotting was done by use of a plasma of a monkey infected with a chimeric virus NM-3rN (Igarashi). Lanes A-D represent the lysates of SHIV-pr (A), SIVmac (B), SIVmac-m D (Dra I-incorporated mutant) (C), and mock infection (D).

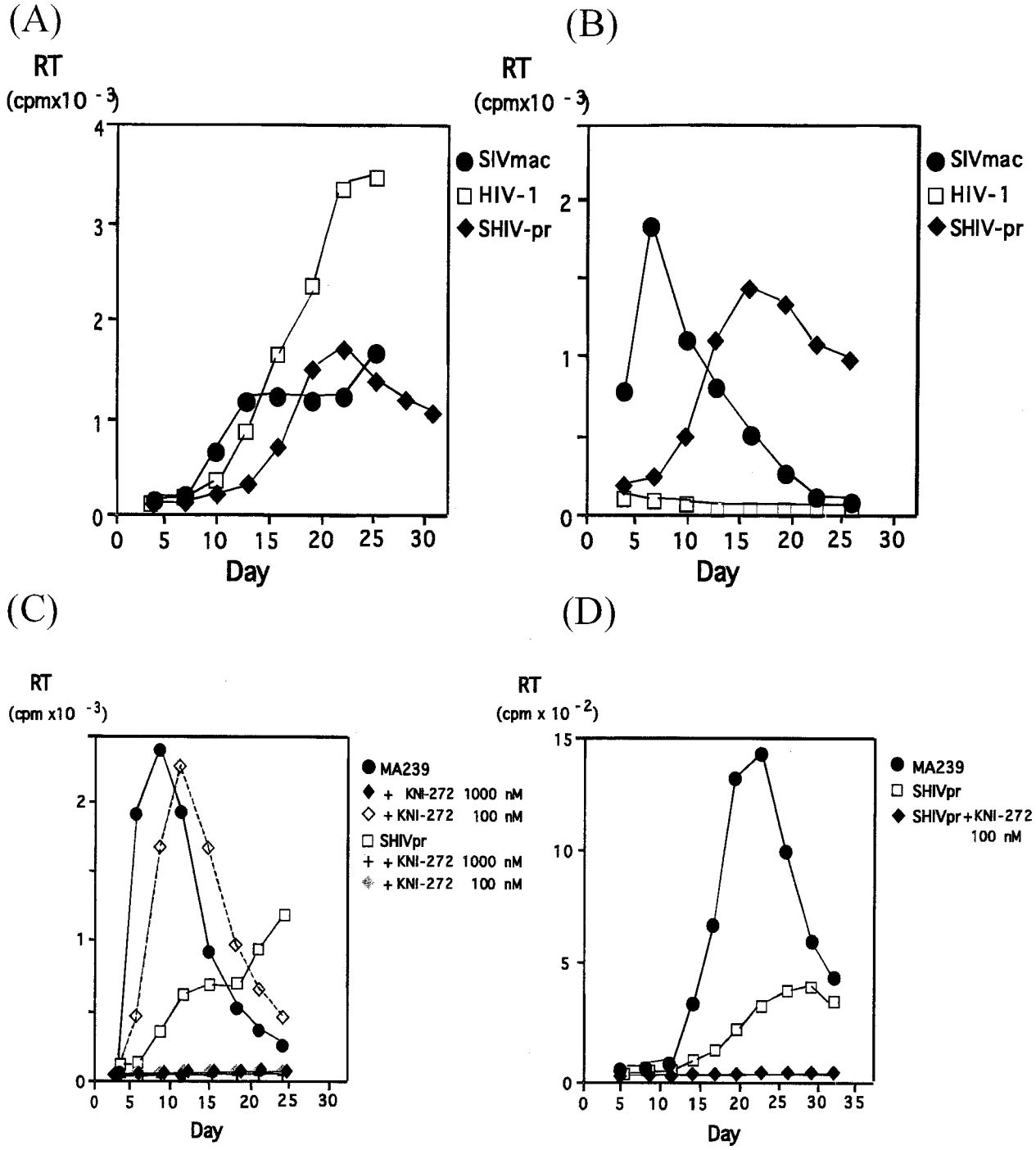

Fig. 3 Growth-kinetics of SHIV-pr and effects of a peptide-analog PR inhibitor, KNI-272. A portion of the stock virus (3-4$\times 10^3$ RT units, equivalent to approximately 1×10^4 $TCID_{50}$) of either SHIV-pr, SIVmac, or HIV-1 was inoculated on to M8166 cells, a human CD4+ lymphoid cell line (A) and HSC-F cells, a cynomolgous monkey CD4+ cell line (B). Viral growth was monitored by measuring the virion-associated RT activities in the culture supernatants which were periodically harvested. Mean values of three independent experiments of growth profiles are plotted. The effects of KNI-272 (o, 100 and 1000 nM) on the viral growth were also examined by measuring the RT acitivities in the culture media. Mean values of two independent experiments of growth profiles in HSC-F cells (C) and rhesus PBMCs (D) are plotted.

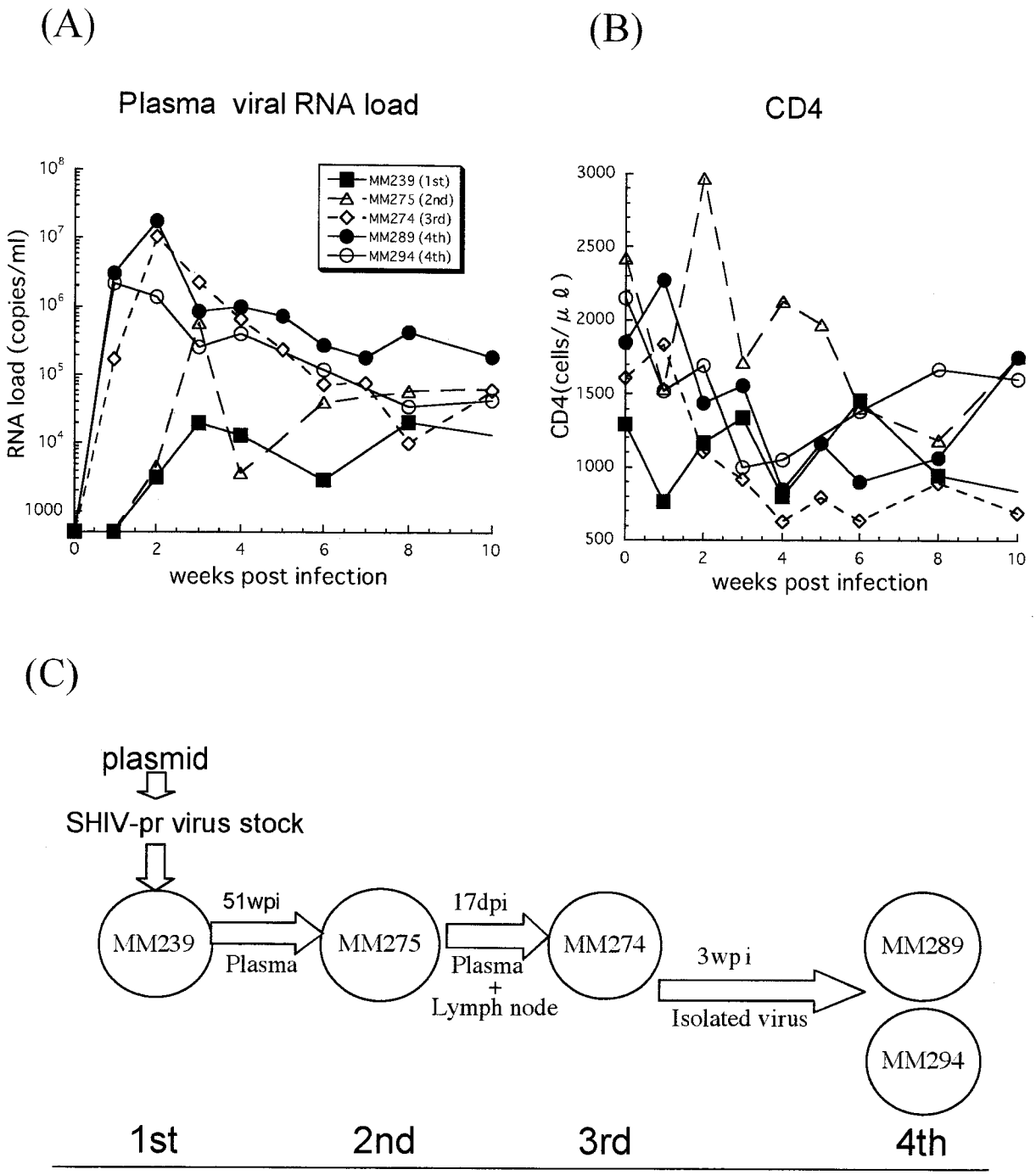

Fig. 4 Enhancement of viral productivity of SHIV-pr by *in vivo* passage. (A) Plasma viral RNA loads (copies/ml) of the respective generation of passages are plotted. (B) CD4 counts (cells/μl) were plotted. (C) A flowchart of *in vivo* passages.

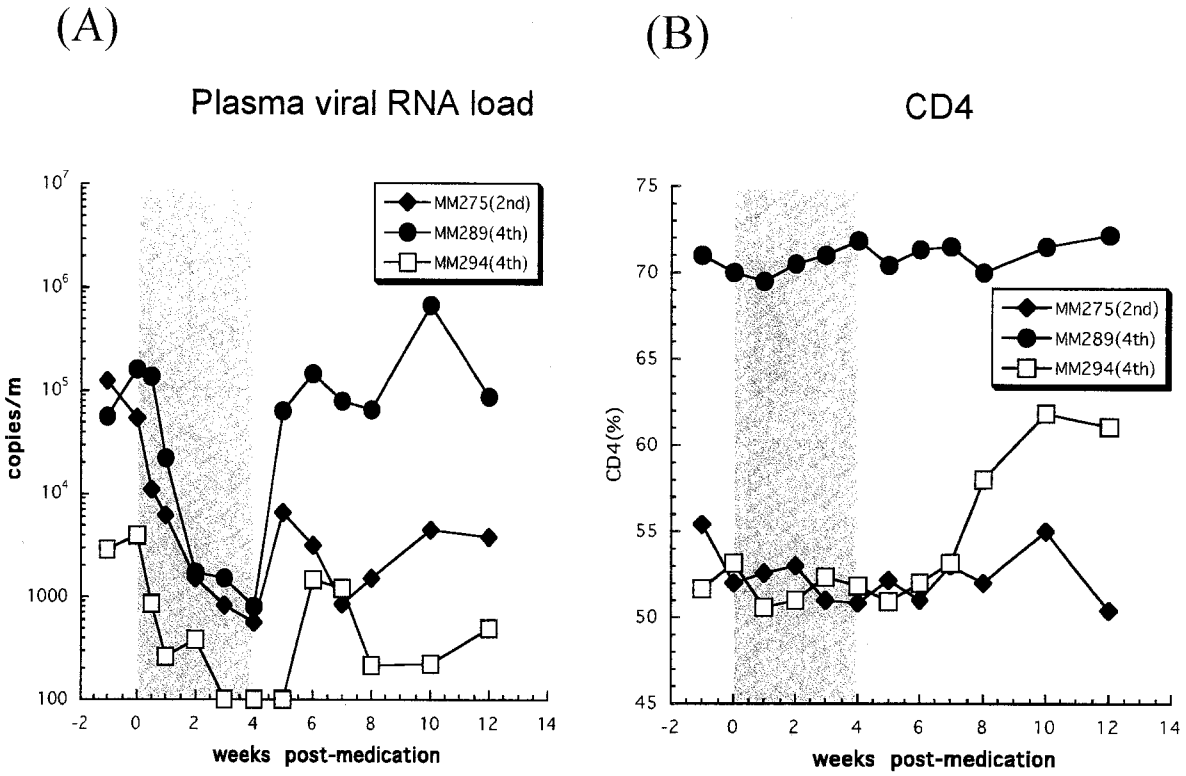

Fig. 5 Medication of SHIV-pr-infected monkeys by Kaletra (a mixture of PR inhibitors, lopinavir and ritonavir, Abbot, Japan). The contents of the Kaletra capsule were suspended in drinking water administered by the oral route (12 mg of lopinavir and 3 mg of ritonavir/kg/day) to 3 monkeys (MM275, MM289 and MM294) for 4 weeks (shaded). (A) Plasma viral RNA load values. (B) CD4 counts.

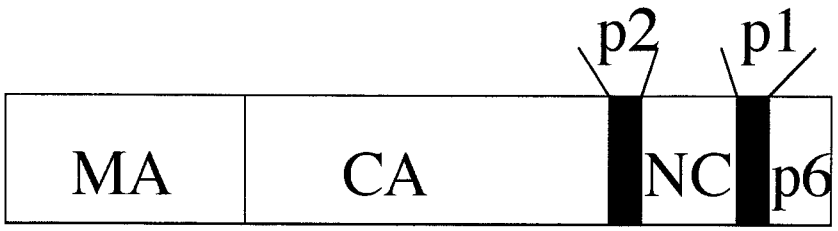

Fig. 6 Schematic representation of the Gag precursor plyprotein. The mature Gag proteins MA, CA, NC, and p6 are shown as open boxes; spacer peptide sequences p2 and p1 are shown as black boxes.

Table 1. Virus isolation and PA antibody titers in SHIV-pr-infected rhesus monkeys (1st generation).

Week	0	1	2	3	4	6	8	11	15	20	27	31	45	51
Virus isolation*														
MM236	−	−	+	−	−	−	−	−	−	−	−	−	−	−
MM239	−	−	+	+	+	+	−	−	−	−	+	−	−	−
PA titer**														
MM236	<32	<32	<32	32	32	32	256	256	256	256	256	256	256	256
MM239	<32	<32	<32	<32	<32	<32	256	1024	1024	1024	1024	4096	≧16384	≧16384

*PA titers were measured by a Genedia HIV-1/2 kit (Fujirebio, Inc., Japan).
**Virus isolation was done by co-culturing with a human T-lymphoid CD4+ Cell line, M8166 cells followed by reverse transcriptase activity assay.

A. Nucleotide

POSITION	212	229
HIV-1	C	G
MM275(2nd)	T	A
MM274(3rd)	T	A
MM289(4th)	T	A
MA239	T	A

B. Amino acid

POSITION	1 10 20 30 40 50 60 70 80 90 99
HIV-1	PQITLWQRPLVTIKIGGQLKEALLDTGADDTVLEEMNLPGRWKPKMIGGIGGFIKVGQYDQILIEICGHKAIGTVLVGPTPVNIIGRNLLTQIGCTLNF
MM275	PQITLWQRPLVTIKIGGQLKEALLDTGADDTVLEEMNLPGRWKPKMIGGIGGFIKVGQYDQILIEICGHKVIGTVLIGPTPVNIIGRNLLTQIGCTLNF
MM274	PQITLWQRPLVTIKIGGQLKEALLDTGADDTVLEEMNLPGRWKPKMIGGIGGFIKVGQYDQILIEICGHKVIGTVLIGPTPVNIIGRNLLTQIGCTLNF
MM289	PQITLWQRPLVTIKIGGQLKEALLDTGADDTVLEEMNLPGRWKPKMIGGIGGFIKVGQYDQILIEICGHKVIGTVLIGPTPVNIIGRNLLTQIGCTLNF
MA239	PQFSLWRRPVVTAHIEGQPVEVLLDTGADDSIVTGIELGPHYTPKIVGGIGGFINTKEYKNVEIEVLGKRIKGTIMTGDTPINIFGRNLLTALGMSLNF

Table2. Viral mutations in the PR domain during the course of *in vivo* passages.

POSITION	276	375	429	433
	CA	p2	NC	NC
SHIV-pr	Arg	Val	Asp	Gly
MM275(2nd)	Lys	Met	Asn	Asp
MM274(3rd)	Arg	Met	Asn	Asp
MM289(4th)	Lys	Met	Asn	Asp
HIV-1	Met	Thr	Arg	Asn

Table 3. Viral mutations in the Gag domain during the course of *in vivo* passages.

POSITION	10	13	33	62	66	71	76	77	82
HIV-1	Leu	Ile	Leu	Ile	Ile	Ala	Leu	Val	Val
Pre treatment	Leu /Phe	Val	Leu	Val	Ile / Phe	Val	Phe	Ile	Ile
After treatment	Leu	Ile	**Val**	Ile	Ile	Val	Phe	Ile	Ile
Pre treatment	Phe	Val				Val		Ile	Ile
After treatment	Phe	Val				Val		Ile	Ile

Table 4. Comparison of amino acid sequences in the PR domain before and after the Kaletra treatment.